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This is to certify that I, Marco Antônio Rochadel, Official Public Translator, designated and installed in Office according to The Official Gazette of June 23, 1982, page 5428, have received and translated, to the best of my knowledge and belief, a document with the following contents:

Ministério da Ciência e Tecnologia

GOVERNO FEDERAL BRASIL PAÍS RICO É PAÍS SEM POBREZA

Ministry of Science, Technology and Innovation – MCT
National Biosafety Technical Commission – CTNBio
Office of the Executive Secretary

comissão técnica nacional CTNBio de biossegurança

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Technical Opinion no. 3964/2014

Proceedings:	01200.002919/2013-77
Applicant:	Oxitec do Brasil Participações Ltda.
CQB:	357/13
Proton:	28300/2013
Matter:	Request for Opinion on Commercial Release of Genetically Modified Microorganism.
Previous Extract:	Number 3676/2013, published on 07.15.2013.
Meeting:	171st Regular Meeting held on April 10, 2014
Decision:	**GRANTED**.

CTNBio, following examination of the proceedings related to the request for Technical Opinion on biosafety of a product intended for commercial release, reached a conclusion favorable to granting the request on the terms of the within Technical Opinion.

Regarding the competences provided by Law no. 11105/2005 and Decree no. 5591/2005, the Commission found that this request complies with CTNBio rules and applicable legislation aimed at securing environment, agriculture, human and animal health biosafety.

TECHNICAL OPINION

ABSTRACT: The person legally in charge of the institution requested CTNBio a technical opinion

related to biosafety involved in the commercial release of the genetically modified *Aedes aegypti* lineage OX513A expressing a conditional lethal treat and a fluorescent marker gene with the purpose of controlling *Aedes aegypti*, the mosquito dengue vector. Oxitec do Brasil Participações Ltda. represents that this request does not contain confidential information. The data provided by the applicant, coupled with the related literature, enable a clear identification of the releasing context, map the threats and characterize possible risks.

I. General Information

The documents included in the proceedings bring all the information necessary to assess the risks that the genetically modified mosquito OX513A may pose to human and animal health. As detailed below, the applicant adequately defines the issues by establishing:

(a) Protection elements and final points of assessment for the safety goals defined by Brazilian legislation;

(b) Biology of the organism;

(c) Characteristics of the probable recipient environments;

(d) Genetic construct and its expression, phenotypic changes and construct stability;

(e) Previous experience with the release of this variety of genetically modified mosquito.

Defining the context of this release leads to identifying different threats, correctly performed by applicant. Each of such threats has the probability of materializing in damage analyzed in this Technical Opinion and classified according to the damage magnitude. With the two above pieces of information for each identified threat, the applicant concludes, to our view in a correct manner, that the risks represented by this mosquito variety to the environment, considered the envisaged conditions of release, are negligible.

In what follows we consolidate, based on the data supplied by applicant and available literature, the representations and conclusions outlined above.

2. Description of the GMO

Two gens were introduced in OX513A mosquito. The first is the tTAV, a system of transcription activation controlled by tetracycline constructed from synthetic DNA based on a fusion of sequences of bacteria *Escherichia coli* and the simple herpes virus (transitional activator of VP16) (Baron *et al.,* 1997). High levels of expression of this transcription factor, which take place in the absence of tetracycline, grant cell lethality. However, in the presence of tetracycline, it links to the tetracycline resistance operon repressor that is part of tTAV, and prevents VP16 transcription.

The second gene introduced in the mosquito is the **DsRed2 marker** gene of the marine coral species *Discosoma*. This is a fluorescent marker extensively used in different species of animals and plants. The expression of this gene produces a red fluorescent protein, and in the OX513A mosquito it takes place at the development stage (larvae and pupas).

Integration of heterologous genes was achieved by using the piggyBac non-autonomous transposon, injected simultaneously with a non-integrating source of piggyBac transposase. Transformation of *Aedes Aegypti* was obtained by micro-injection of individual embryos. Two plasmids were coinjected: plasmid **513 PB Red teto-tTAV** and **256 piggyBac Helper**. The result was an integration of the heterologous genes in a single site. The only copy inserted in the genome was fully characterized and shown to be stably maintained.

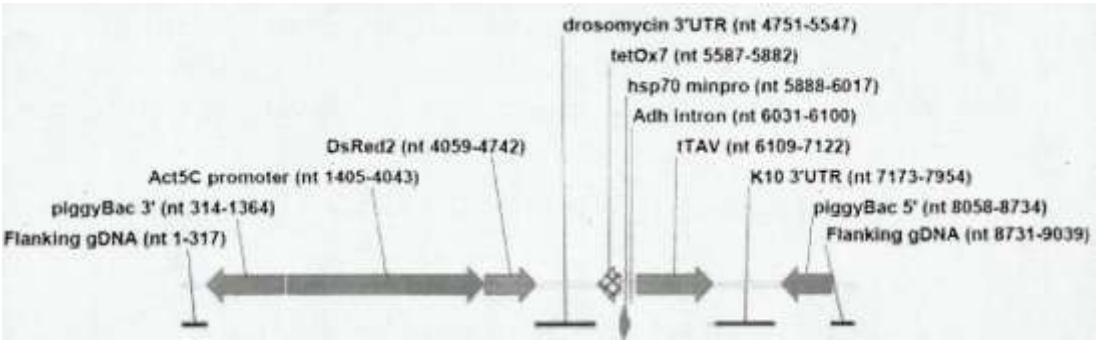

Figure 1: The construct elements inserted in the genome and confirmed by sequencing are shown in Figure 19 of the first volume of the commercial release dossier

Protein DsRed, constitutively produced, enables detection of the event with the use of a UV lamp. Protein tTAV, lethal in high cell concentrations, is controlled by the tTAV system described in the text.

3. Product Biosafety

GMO Analysis according to Ruling Resolution no. 5, of March 12, 2008, Annex III.

The genetic *Aedes Aegypti* construct OX513A involves conditional expression of a lethal gene. Construct stability was fully demonstrated in the documentation and was expected, given the characteristics of the vector used and the type of insertion. Studies submitted by the company and already partially published show that:

- The sequence of the *Aedes Aegypti* construct OX513A is exactly as intended;
- The lineage has no sequences of the plasmid structure used in the transformation;
- A non-autonomous transposable element was used in the transformation and showed to be stable under a wide variety of conditions;
- The insertion is stable for several generations and follows the relations of expected Mendelian inheritances.

The gene expression seems to display discrete fluctuations, probably due to a partial penetrance of the transgene. However, this characteristics fails to affect the behavior of the released GM mosquito population, since lethality is assured for most part of the progeny of escapes, leading to a sharp reduction in population and elimination through competition with non-modified mosquitos, by predation and by other inhibiting mechanisms that are present in the environment, including anthropic mechanisms (Williams *et al.* 2010). In general, the applicant failed to record any biologically relevant difference between the behavior and phenotype of OX513A and that of the non-modified counterpart, except for the reduction in survival of larvae and longevity of OX513A adults when compared with the non-modified element (result expected by genetic modification), when the following parameters were assessed in different conditions of temperature and humidity:

- Reproductive ability;
- Response to different temperature schemes;
- Survival;
- Lineage longevity with and without tetracycline;
- Lethality phenotype penetrance in a range of tetracycline dosage;
- Susceptibility to chemical insecticides.

The applicant studied existence of relevant differences in mating and competitiveness behavior of the OX513A lineage contrasted with populations in the field or the feral type of *Aedes Aegypti* over the world (Dossier, book 3, section 2.2.3). Data were provided on inter- and intra-specific mating also in Annex II, Section 16.1.4. There is no feral kin of *Aedes Aegypti* in Brazil with which OX513A may interchange gametes.

***Aedes Aegypti* biology**, widely documented, favors the biosafety of this product: the insect only mates with other insects of the same species and displays dispersion limited to urban environments, though it may be transported for long distances in land vehicles, watercrafts and even in airplanes. In such casual transportation, the non-modified mosquito may find an opportunity to colonize new urban niches, yet never to invade sylvan environments. Another important biologic aspect that is stressed in the dossier and supported by the literature, is the origin of this mosquito species: in Brazil it is an exotic species, restricted to cities. Therefore, we reach the conclusion that the possible commercial release fails to include valued species (except for humans) or ecosystem services that may be affected by the GM mosquito, since there are no behavioral differences between the GM and conventional insects and they do not impact the urban fauna in a significant way.

Previous experience with the release of this variety of GM mosquito

Although there is no previous commercial release experience with this GMO, there is a considerable amount of pertinent information coming from planned release of this mosquito in other countries (Lacroix *et al.*, 2012 and dossier data) **and in Brazil**. The data submitted,

distributed across the dossier scattered in different topics that were treated in compliance with Ruling Resolution nº 5, confirm that the mosquito biology and phenotypic changes coming from the genetic transformation suggest that apparently there is no impact of OX513A in the environment. Despite considerations of non-transportability of data generated in different environments, it is quite clear that the synanthropic and urban characteristic of the population of this mosquito species facilitates the transference of data generated in other countries.

Threats identified by applicant

Table 2 of item 2.1 (Identification of protection goals and assessment outcomes) of Book 2, Annex III, lists the threats initially identified by applicant and show data and arguments to conclude that the risks are insignificant. Threats to human health, on our view correctly identified, were:

- The genetic modification could have introduced toxic or allergenic proteins in the *Aedes Aegypti* OX513A mosquito, especially in its saliva;
- Vector competence to transmit diseases to the human being could have been changed towards greater transmission efficiency.

Threats identified for other institutions and organizations

A concern voiced in some circles is that introduction of a new lineage of *Aedes Aegypti* could lead to transmission of other diseases or even to a change in transmission mode.

A concern that is repeatedly brought to the attention of CTNBio is that of tetracycline residues in served waters and sensibility of event OX513A to the presence of tetracycline. Sensibility to tetracycline was deeply studied by applicant. Survival of the OX513A event reduces to <5% in the absence of tetracycline because of expression of the lethal gene tTAV. Survival of progeny resulting from the mating of a male OX513A and local *Aedes Aegypti* females is equally reduced, since the tTAV expression in the larva enables accumulation of the protein that interrupts cell processes (Gill & Ptashe, 1988; Lin *et al.*, 2007). In order to identify the lesser

concentration of tetracycline, enabling greater survival of *Aedes Aegypti* OX513A heterozygotes when contrasted to the ones raised in the absence of tetracycline, the response of the lineage to different doses of tetracycline was evaluated. According to the dossier, OX513A larvae raised under tetracycline concentrations equal to or lower than 1ng/ml failed to generate a higher percentage of adults than that of larvae raised in the absence of tetracycline (0 µg/ml) (p= 0.212). A review of the literature indicated that the maximum concentrations reported in field sites around the world were recorded as ranging between 110pg/ml and 970pg/ml (Brown *et al.*, 2006; Le-Minh *et al.*, 2011; Sarmah *et al.,* 2006). This shows to be improbable that the larvae of the OX513A event may find tetracycline concentrations in the environment that are high enough to increase the number of functional adults above 5% (taking into consideration a characteristic of incomplete penetrance of the lethality trace). Based on the literature, one reaches the conclusion that it is highly improbable to find concentrations of tetracycline above the redemption level of 1ng/ml in the typical breeding places of *Aedes Aegypti* (Le-Minh *et al.*, *op. cit.*). Since the presence of tetracycline will always be lower that the necessary to suppress lethality, and that the *Aedes Aegypti* prefers to lay its eggs and develop in cleans waters, this question becomes irrelevant in assessing the GMO risk, although it may be important for global control strategies. Even so, one shall keep in mind that the pre-commercial monitoring phase of the process will include the monitoring of the antibiotic in the environment.

Risk characterization

The first threat identified involves the potential toxicity or allergenicity of the recombinant protein in the insect's saliva. Although the number of females released may be relatively small against the population of non-GM mosquitos of the same gender, there is a slight possibility that an individual be bitten several times by the GM insects along the period in which the vector is under attack. Allergic reactions could be severe, depending on the allergenic potential of the protein. However, the applicant conducted a thorough study by bioinformatics and

rightly concluded that protein fails to exhibit allergenic potential. Therefore, though there is a small probability of an individual being repeatedly bitten by female GM mosquitos, the protein is not allergenic and the damage is null.

Regarding the threat of transmission of new diseases or changing transmission patterns due to genetic modifications of the vector, considering that:

- The population of OX513A *Aedes Aegypti* tends to reproduce rapidly after the release of male mosquitos, therefore preventing this population to adapt to new vector functions;
- A small percentage of females is released (which may be further reduced, in case of need, with the adoption of new technologies – e.g. Marois *et al.*, 2012);
- There are no records in the literature related to adaptation of vectors to new agents caused by changes introduced by intended genetic modifications in the vector (though natural changes in dengue virus lineage modulate the vector ability – Christofferson and Mores, 2011);

we may conclude that the threat carries no hypothesis that support a paths to possible damage, being either null or very low the probability that damage will come true.

Risk classification

Classification of risks depends on existence of concrete paths leading from threats to consequent damages, since these paths define both probability and magnitude of damages. In this case, regarding the aspect of environmental safety, there are concretely no elements of protection possible of definition in the context of the problem that may minimally avail a route to plausible damage. Hence, we conclude that the risks may be classified as insignificant or negligible regarding presence of the same non-genetically modified insect.

As regards the three threats identified, either there is no plausible path to the possible damage

or there is only a remote probability of it. For the foregoing we conclude that the risks represented by direct action of the GMO and that are different from those risks observed in the non-genetically modified species are either insignificant or null.

Environment Safety

Protection goals in probable receiving environments: Brazilian legislation determines widely that the environment shall be protected, including man and its livestock, and pet animals. Applicant adopted a methodological strategy of identifying the probable receiving environments and, through analysis of its characteristics, select targets of protection. This way, the applicant rightly identified that the environment, in the case of release of the OX513A variety, is essentially restricted to the urban and suburban environments. Indeed, all data available in the literature point towards a synanthropic trait of *Aedes Aegypti*, restricted to urban and suburban environments without any potential of establishing in sylvan or farm environments, and without vector ability for other species, except man, at least in the urban environment.

In the urban environment, the main target of protection is clearly the human being itself, an element inseparable from the environment. Besides, the potential impacts on some insectivorous organisms were also assessed. In our understanding, an urban environment is quite restricted in these aspects and the population of GM insects tend to reduce rapidly; therefore, the study conducted by the company on Section 2.9 of Book 2, Annex III of the dossier, is not interesting for a risk assessor: the models examined by applicant are classically used in situations where certain fauna elements may be impacted, but we do not think they are relevant and only add scientific information of academic interest. However, assessment of these parameters resulted from mandatory compliance with the requisites of Assessment of Human and Animal Health appearing in Ruling Resolution no. 5.

Aedes Aegypti is a tropical cosmopolitan invasive species living between latitudes 40^{o} N and

40° S, limited by temperatures below 15 to 10 °C, when the insect becomes unable to fly and moves its members in a slow way, decelerating considerably its development time (Christophers, 1960). Climate, urbanization, water storage and availability of breeding spots are the main factors influencing the *Aedes Aegypti* distribution. Therefore, dispersion of the mosquito depends on stored water or presence of water in artificial recipients, on the presence of human hosts and passive transportation mediated by humans, as well as availability of breeding spots. Implications for the insect biology, resulting from insertion of transgenes, was analyzed (relevant differences in mating behaviors and competitiveness of OX513A lineage with field populations or with the sylvan type of *Aedes Aegypti*. Data on inter- and intra-specific mating were discussed, and found that there is no sylvan kin of *Aedes Aegypti* in Brazil with which OX513A may interchange genes. *Aedes albopictus* (Skuse) and *Aedes Aegypti* (L.) belong to different taxonomic divisions of the large subgenus Stegonyia and are native of Asia and Africa, respectively. However, both species are invasive and may be found living side by side in some environments. Consequently, it is probable that virgin females of both species find heterospecific males attracted to the same hosts, and interspecific mating might be a possibility.

However, mating of *Aedes Aegypti* is extremely specific to the species, although Tripet *et al.*, (2011) had recently reported interspecific mating in field populations of *Aedes Aegypti* and *Aedes albopictus* in Florida, though in a reduced rate (1.6%). This was also reported by Nasci *et al.*, (1989). Mating behavior is also discussed below in this opinion.

Although not directly related to the GMO impact in the environment, a potential increase of *Aedes albopictus* populations by a selective reduction of *Aedes Aegypti* has been aired as a disadvantage to the controlling process through RIDL (*cf.* report of Dr. José Maria Ferraz on a visit to Moscamed). Though populations of both species may be sympatric, this occurs only in small forest/urban area transition strips (Honório *et al.*, 2009) and scantily in the surroundings of densely arborized areas of cities. There is, in fact, a clear spatial preference by one or other

species (Duncombe *et al.*, 2013). Therefore, an improbable and eventual fluctuation of populations of *Aedes albopictus* due to elimination of *Aedes Aegypti* is entirely irrelevant from dengue controlling viewpoint. Indeed, consensus indicate that *Aedes algopictus* is essentially a sylvan species and is present in cities solely in the vicinity of woods or extensive gardens.

Mating behavior of OX513A event males was studied in comparison with the sylvan non-modified type (Bargielowski *et al.*, 2011) and remained verified that, during a life cycle, GM mates inseminated less females, but the number of females inseminated during the first three days was similar for males of the GM and non-GM lineage. Concerns about copula preference and polyandry, stressed as important in the Moscamed visit report by José Maria Ferraz, but deemed as little relevant on the reports on GMO risks, is the possibility that females copulate more than once. Anyway, this question is adequately answered by the literature: there may be more than one copula, though virgin females search for partners more rapidly and efficiently; besides, it seems that an efficient retention of semen does not happen in the second copula, as shown more than one decade ago by Spielman and his colleagues (1967). The current consensus is that the polyandry potential among *Aedes Aegypti* females is very low. (Helinski *et al.*, 2012). Additionally, the results obtained in significantly reducing the native population after preliminary releases of OX513A, jointly with other recent studies, corroborate the low polyandry potential and indicate that ability of mating of GM mosquitos compared with the sylvan type is similar, which verify the potential control of the proposed strategy (Harris *et al.*, 2012; Massonett-Brunnel *et al.*, 2013; Lee and colleagues, 2013).

From the viewpoint of environmental risk assessment, this request has no particular challenges, truth being that it is notably simpler than those related to the majority of transgenic plants, mainly because the conditionally lethal character of the OX513A *Aedes Aegypti* and its biology, being an exotic species, reproductively isolated and with exclusive urban distribution in Brazil. Even so, all possible concerns related to direct risks of introducing the GMO in the environment brought to light by documents forwarded to CTNBio, by active

search in publications and by applicant, were treated in previous opinions and consolidated in this opinion.

4. Post-Commercial Release Monitoring Plan

Following the commercial release, monitoring shall be conducted in places where the OX513A lineage was released, in three representative points, in order to assess the population of *Aedes Aegypti* and the proportion of the population carrying the OX513A transgene (fluorescent marker). Traps will be monitored on a monthly basis. After 12 months of release and beyond, at each year, monitoring will be conducted to assess stability of the genetic marker. The use of tetracycline in Brazil will also be monitored through analysis of the literature and research reports generated by residual waters treatment plants, enabling the analysis of any change in use and in levels of tetracycline in the environment.

This commission holds the opinion that monitoring of population levels of the *Aedes albopictus* mosquito shall be included in this plan.

5. Final Opinion

Pursuant to provisions of Law no. 11105 and Ruling Resolution no. 05, CTNBio is in charge of assessing risks, limited to the direct biologic risks resulting from releasing a GMO in the environment. Accordingly, this opinion does not focus issues of technology efficacy, costs and advantages/disadvantages as against other technologies of *Aedes Aegypti* population control.

Finally, questions directly linked to dengue control are not a CTNBio concern, since the matter is under the Brazilian Health Ministry and State Secretariats that may select adopting the technology to control this endemic disease. Moreover, statistical tools and experiments to assess technology impact in dengue control are completely different from those required to risk assessment and, therefore, the data presented, surely sufficient for CTNBio to assess the risk attached to the GMO impact, cannot be extended or used in dengue control analysis. One shall keep in mind that specialists have always looked for, and still do, new and effective control mechanisms related to the vector as the most feasible of controlling the disease until an efficient vaccine becomes a reality. Currently, controlling the vector is pursued with

elimination/reduction of breeding sites and use of insecticides. Therefore, in this context, the technology described in this document would come as an option to reduce the vector population.

We may conclude, therefore, based on the evidence submitted by applicant, related literature and our risk assessment, that the mosquito *Aedes Aegypti* poses no additional risks to the environment, human beings and animals when compared to the same, genetically modified, species. Our opinion is thus favorable to the release sought.

6. Dissenting Opinion (Request for Examination)

At the 170th Plenary CTNBio Meeting, held on March 13, 2014, Dr. Leonardo Melgarejo and Dr. Antônio Inácio Andrioli requested examination of the proceedings and submitted their opinion on the 171st Regular CTNBio Meeting of April 10, 2014, where they concluded that:

"Summarizing, contrary to the opinions favorable to the request for commercial release of OX513A, we examined a probable damage path, not adequately contemplated in the proceedings. It relates to the damage that may become real through re-emergence of viral human and/or animal epidemics of zoonotic origin, or otherwise, previously, or otherwise, to the release in large scale of OX513A, with significant degradation of public health in these areas and potential negative social and economic consequences in municipalities affected. The path shall be materialized by occupation of the Aedes Aegypti ecological niche by A. albopictus – as a result of the large scale release of OX513A – associated to a change in the epidemiologic profile of animal, human and zoonotic viruses, providing them greater ineffectiveness, through a change in the vector and/or going around the immunologic barriers of secondary vectors. In this context, in circumstances aggravated by lack of compliance with the legislation in effect; by inexistence of assessment protocols adequate to risk analysis involving a flying insect; by insufficiency of studies submitted; by lack of inclusion of final results of filed studies passed by CTNBio; and considering that the commercial release of OX513A, in such conditions, poses relevant and irreversible risks to health and environment, in our opinion with a high to moderate probability of occurrence, we recommend that the process be put in

DILIGENCE until its completion, and that it is returned to be analyzed pursuant to rules to be established by CTNBio."

The above opinion was overruled by the majority of votes.

7. Bibliography

Baron U., Gossen, M., and Bujard, H., (1997). Tetracycline-controlled transcription in eukaryotes: novel transactivators with graded transctivation potential. Nucleic Acidsd Research 25, 2723-2729.

Christofferson, R.C., Moraes C.N., Estimating the magnitude and direction or altered arbovirus transmission due to viral phenotype. PLoS One. 2011 Jan 27; 6(1):e16298.

Lacroix, R., McKemey, A.R., Raduan, N., Kwee Wee, L., Hong Ming, W., Guat Ney t., Rahidah A. A. A., Salman, S., Subramaniam, S., Nordin O., Anum, A. T. N., Angamuth, C., Marlina Mansor, S., Lees, R. S., Naish, N., Scaife, S., Gray, P., Labbé, G., Beech, C., Nimmo, C., Alphey, L, Vasan, S. S.,Han Lim, L., Wasi, A. N., Murad, S., Open field release of genetically engineered sterile male *Aedes Aegypti* in Malaysia, PLoS One. 2012;7(8):e42771.

Marois, E., Scali, C., Soichot, J., Kappler, C., Levashina, E. A., Catteruccia, F. High-throughput sorting of mosquito larvae for laboratory studies and for future vector control interventions. Malar, J., 2012 Aug 28; 11:32.doi: 10.1186/1475-2875-11-302.

Williams D.R., Bader C.A., Kerney, M.R., Ritchie, S.A., Russell, R.C., The extinction of dengue through natural vulnerability of its vectors. PLoS Negl Trops Dis. 2010 Dec 21; 4(12).

Dr. Edivaldo Domingues Velini
CTNBio President

In Witness Whereof, I have hereunto set my hand and seal in this City of Brasília, Federal District, Brazil, this Wednesday, July 16, 2014.

Fees according to

Official Gazette of 04/15/2011 Marco Antônio Rochadel

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